Survey of Selected Antibiotic Resistance Genes in Agricultural and Non-Agricultural Soils in South-Central Idaho

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Abstract

Improving our understanding of antibiotic resistance in agroecosystems is important for the protection of human, animal, and ecological health. In south-central Idaho, antibiotic resistance genes (ARGs) [$bla_{\text{CTX-M-1}}$, *erm*(B), *sul1*, *tet*(B), *tet*(M), and *tet*(X)] and a class 1 integron-integrase gene (*intI1*) were quantified in agricultural and non-agricultural soils (96 total sites) under various land use practices (cropland, forestland, inactive cropland, pastureland, rangeland, recreational, residential). We hypothesized that gene occurrence and abundance would be greater in intensively managed agricultural soils. The ARGs (except $bla_{\text{CTX-M-1}}$) and *intI1* gene were detected in many of the soils (15 to 58 out of 96 samples), with *sul1* and *intI1* being detected the most frequently (60% of samples). All of the genes were detected more frequently in the cropland soils (46 sites) and at statistically greater relative abundances (per 16S rRNA gene) than in soils from the other land use categories. When the cropland gene data was separated by sites that had received manure (27 sites), it was revealed that the genes [except *tet*(B)] were found at statistically greater abundances (7- to 22-fold higher on average) than in soils that were not treated. The results from this study provide convincing evidence that manure use in Idaho cropland soils increases the expansion of antibiotic resistance-related determinants.

Keywords: Agroecosystem; Antibiotic resistance gene; Class 1 integron; Cropland; Manure; Soil

Introduction

Agriculture produces food, fiber, and fuel to satisfy the demands of an ever-growing world population. However, like many anthropogenic activities, there are a number of negative environmental impacts associated with agricultural production and its intensification (Tilman *et al.*, 2002). Some of the most prominent issues include soil degradation, water and air pollution, deforestation, and climate change. Recently, attention has also been given to prophylactic and therapeutic uses of antibiotics in food-

animal production and their potential contribution to the development of drug resistant bacteria *in-vivo*, during manure storage, and in soils receiving manure solids or wastewater as a fertilizer (Binh *et al.*, 2008, Negreanu *et al.*, 2012, Chantziaras *et al.*, 2014). The continued use of large quantities of antibiotics in animal production raises concerns about the release of these drugs and the increasing prevalence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment. While use of antibiotic drugs can enrich ARB, the interplay between antibiotic drug use, ARB/ARGs, and land use practices in agroecosystems is poorly understood (Williams-Nguyen *et al.*, 2016).

Samples analyzed from isolated environments have demonstrated that antibiotic resistance is a natural and ancient phenomenon that predates clinical antibiotic use (Miteva *et al.*, 2004, D'Costa *et al.*, 2011, Bhullar *et al.*, 2012). Antibiotic resistance genes occur in native soils without anthropogenic selection pressure (Allen *et al.*, 2009), but evidence suggests that their abundance in agricultural soils has been increasing since the 1940s (Knapp *et al.*, 2010). Although the environmental or anthropogenic causes have not been determined, the gene level increases occurred during the same period when industrial production of antibiotics was growing rapidly. Furthermore, agricultural intensification increased at the same time and antibiotics were used for growth promotion purposes, which ultimately made their way to agricultural fields via manure application (Davies & Davies, 2010). Manure application can transfer ARB and ARGs to soils, as well as antibiotic residues and other xenobiotic compounds, resulting in the expansion of antibiotic resistance reservoirs when compared to that of native soils (Heuer & Smalla, 2007, Cytryn, 2013, Amarakoon *et al.*, 2016, McKinney *et al.*, 2018).

The detection of ARGs in soils, manures, and agriculturally impacted environments is well documented in the scientific literature, but the risk of elevated ARG levels on public health is not well understood. Antibiotic resistance genes are genetic code used by ARB to make proteins to resist the effects of antibiotics, with ARGs often thought of as being comparable to “emerging contaminants” (Pruden *et al.*, 2006, Dalkmann *et al.*, 2012). Bacterial species that can resist the activity of one or more antibiotic compounds as a result of their inherent structural or functional characteristics are considered “intrinsically resistant”. These inherent properties predate the antibiotic era and are chromosomally encoded or occur because bacteria lack pathways or target site (Franklin *et al.*, 2016). In contrast, “acquired resistance” occurs when bacteria horizontally obtain ARGs through conjugation (cell-to-cell

mediated), transformation (uptake of naked DNA), or transduction (phage mediated). Conjugation uses mobile genetic elements, such as transposons plasmids, which all play a critical role in the development and dissemination of antibiotic resistance among clinically relevant organisms (Allen *et al.*, 2010). In the case of transformation, only a small number of clinically relevant bacteria are known to be able to incorporate naked DNA to develop resistance (Munita & Arias, 2016), whereas transduction is not considered to contribute to gene exchange among distantly related bacteria (Dröge *et al.*, 1999). Regardless of gene acquisition mechanism, there is little information available concerning the frequencies at which ARGs are transferred in agricultural environments (Durso & Cook, 2014).

The first step of many ARG investigations generally involves the extraction of genomic DNA from environmental samples using commercially available kits, followed by quantitation of selected genes using a PCR-based approach (Pruden *et al.*, 2012, Agga *et al.*, 2015, Rieke *et al.*, 2018). The ARGs detected in such studies are both intracellular and extracellular, as methods to distinguish between the two are not regularly used (Zhang *et al.*, 2013). In addition, it is not known if the ARGs are associated with viable bacteria and being expressed or have the ability to cause resistance in recipient bacteria. Despite some of these known limitations, monitoring ARGs via a PCR-based approach can provide valuable information on the proliferation of antibiotic resistance in an ecosystem (Luby *et al.*, 2016). An increase in ARG levels is a general indicator that ARB enrichment is occurring and/or selective agents (e.g., antibiotics, metals), ARB, and ARGs are being released into the environment. Although, it could also simply reflect changes in the bacterial community composition. The primary aim of this study was to determine the occurrence and abundance of selected ARGs [$bla_{\text{CTX-M-1}}$, *erm*(B), *sul1*, *tet*(B), *tet*(M), and *tet*(X)] and a class 1 integron-integrase gene (*intI1*) in soils under various agricultural and non-agricultural land use practices (i.e., cropland, inactive cropland, pastureland, rangeland, recreational, forest, residential) in south-central Idaho. Compared to non-agricultural lands, croplands are routinely exposed to manures, treated with synthetic fertilizers, planted with various crops, and subjected to irrigation and tillage practices. As a result, we hypothesized that the gene targets would be present at greater frequencies and relative abundances in intensively managed agricultural soils compared to soils under the other land uses.

Materials and Methods

Description of Region and Sampling Sites

South-central Idaho is an intensively managed region with a high percentage of the land in irrigated row crops and animal production. This region has as a semiarid climate and consists of hot dry summers and cool wet winters, with a mean annual temperature of 8.7°C and precipitation of 284 mm. Idaho is currently the fourth largest dairy state in terms of milk production, with a total of 602,000 lactating cows (NAS, 2018). Approximately 71% of these dairy cattle are located in 6 counties within south-central Idaho. There are also about 93,000 beef cattle in this region, with stockers/cows often grazed on non-irrigated rangeland and fields after harvest and feeder cattle in feedlots. The manure solids (stacked and composted) and wastewater from the manure management systems, especially for dairy production, are applied to the surrounding cropland in the fall or spring. In addition to cattle manure, some crop fields also receive biosolids from municipal wastewater treatment or aquaculture facilities.

We collected information regarding the crop/vegetation, irrigation, presence of animals, and organic fertilizer treatments at each sampling site. This information was provided to us by the land owners or farm supervisors and/or deduced by a visual inspection of the land when necessary. At some sites it was not possible to acquire information about current and past management practices, so at the minimum, each sampling site was categorized based on the land use and land cover classification system according to Anderson (1976)). The land use categories (followed by number of soil samples) included cropland (46), inactive cropland (6), pastureland (8), rangeland (17), recreational (13), forestland (4), and residential (2). Cropland is irrigated land where row or field crops are grown; inactive cropland is land that has been taken out of agricultural production; pastureland is land that is managed as pasture areas for livestock grazing; rangeland is land where the potential natural vegetation is predominantly grasses, grass-like plants, forbs, or shrubs, which may have been subjected to grazing activities; recreational land areas include city, county, and state parks which are open to a variety of recreational activities; forestlands are any lands covered by woody vegetation and support timber harvest and many kinds of outdoor recreation; and residential land areas support single-family residences in rural settings. Information about the sampling sites is located in supplementary Table S1.

Collection of Soil Samples

Soil samples were obtained from 96 sites within 7 counties (i.e., Twin Falls, Jerome, Gooding, Lincoln, Blaine, Minidoka, and Cassia) between 4 Aug and 30 Nov, 2016. Using an ethanol-disinfected auger, soil samples were collected from the top 15 cm after removing as much of the organic residue as possible. The samples were placed in clean sealable plastic bags and immediately stored in a cooler with ice packs during transport to the laboratory. Upon arrival at the laboratory, the soils were processed using a 2-mm sieve, then returned to the plastic bag for storage at -75°C until analyzed.

Soil Chemical Analysis

The moisture content of each soil was determined by oven drying overnight at 105°C. Air-dried and sieved soil (2 mm) were analyzed for pH and electrical conductivity (EC) using a 1:1 soil:deionized water suspension. All soils were pulverized using a mortar and pestle, then a 50-mg sample was analyzed for total carbon and nitrogen in a Flash EA1112 NC Analyzer (CE Elantech, Inc., Lakewood, NJ, USA). The pulverized soil was analyzed for inorganic carbon using a modified pressure-calcimeter method (Sherrod *et al.*, 2002). Soil organic carbon was determined as the difference between total and inorganic carbon. The soil properties are located in supplementary Table S1.

Quantitation of Genes

Total DNA was extracted from approximately 500 mg of soil (wet wt.) using the FastDNA Spin Kit for soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA) following the manufacturer's protocol. The DNA extracts were stored in DNase/pyrogen-free water at -20°C until analyzed by quantitative real-time PCR (qPCR) on a iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each individual reaction consisted of 12.5 μL of 2× SsoAdvanced™ Universal Probes Supermix (Bio-Rad), 250 nM of forward and reverse primers and probes, 2 μL of DNA template (10-fold diluted in molecular biology grade water to minimize PCR inhibition), and sterile DNase/pyrogen free water to create a final volume of 25 μL. The gene targets were 16S rRNA, $bla_{\text{CTX-M-1}}$, *erm*(B), *intI1*, *sul1*, *tet*(B), *tet*(M), and *tet*(X). Primers, probes, annealing temperatures, amplicon lengths, and sequences can be found in Dungan *et al.* (2018)). The thermocycler conditions consisted of one cycle at 95°C for 3 min, 40 cycles at 95°C for 15 s, and annealing temperature for 30 s. The qPCR runs included a standard curve

covering seven orders of magnitude, and each sample was analyzed in duplicate. Standards were created using gBlocks Gene Fragments (Integrated DNA Technologies, Coralville, Iowa, USA).

Statistical Analysis

To determine the relationship between the ARG and *intI1* relative abundances in soil, Pearson correlation coefficients (*r*) were calculated using the CORR procedure in SAS (version 9.4, SAS Institute Inc., Cary, NC). Relative gene abundance data (gene copies/16S rRNA gene copies) was log transformed before analysis to meet assumptions of normality and homogeneity of variance. Highly correlated values were defined as $r \geq 0.70$. To compare the relative abundances of ARGs and *intI1* between cropland and all other soils and within cropland soils (manure versus no manure), the Wilcoxon rank-sum test was performed using the NPAR1WAY procedure in SAS. All statements of statistical significance were declared at $P < 0.05$. The hierarchical cluster analysis was performed to compare relative gene abundances among individual sites using the hclust function in R (version 3.4.3, R Core Team) with complete linkage and square Euclidean distance.

Results and Discussion

The absolute abundance of ARGs and *intI1* and 16S rRNA genes in the soils is presented in Fig. 1. All of the ARGs (except $bla_{CTX\text{-}M\text{-}1}$) and *intI1* were detected in some of the soils (15 to 60 detections out of 96 samples), with *sul1* and *intI1* being detected the most frequently (60% of samples), while *erm*(B), *tet*(B), *tet*(M), and *tet*(X) were detected less frequently (16 to 45%). Regardless of land use category, the median number of gene copies/g soil (dry wt.) were: *erm*(B), 1.1×10^5; *sul1*, 1.6×10^5; *tet*(B), 5.2×10^4; *tet*(M), 1.4×10^5; *tet*(X), 7.5×10^4; and *intI1*, 8.4×10^4. The 16S rRNA gene was detected in all soils as expected, at a median level of 9.2×10^9 gene copies/g soil. The ARGs targeted in this study were chosen because they include resistance to antibiotics that are considered medically important (WHO, 2016), utilize different resistance mechanisms, and cover a wide range of bacterial hosts (van Hoek *et al.*, 2011, Roberts & Schwarz, 2016). These genes have been targeted in two previous studies of ours (Dungan *et al.*, 2018, McKinney *et al.*, 2018) and in many other soil-focused studies (Fahrenfeld *et al.*, 2014, Marti *et al.*, 2014, Kyselková *et al.*, 2015, Nõlvak *et al.*, 2016, Sandberg & LaPara, 2016). Despite the fact that community DNA in the present study (and above mentioned studies) was obtained from both live/dead

soil bacteria and extracellular forms, ARGs and the *intI1* gene are considered useful indicators to evaluate the antibiotic resistance status in environmental settings (Berendonk *et al.*, 2015).

The $bla_{\text{CTX-M}}$ genes encode resistance to β-lactam antibiotics, with CTX-M enzymes being the most prevalent extended-spectrum β-lactamases worldwide (Canton & Coque, 2006). Because CTX-M-1 group enzymes are not currently endemic in the U.S., the absence of $bla_{\text{CTX-M-1}}$ in all soil samples was not unexpected. The *erm*(B) gene confers resistance to macrolide–lincosamide–streptogramin B (MLS_B) antibiotics and is found in a wide host range (many genera of aerobic and anaerobic Gram-positive and Gram-negative bacteria) compared to numerous other MLS_B resistance genes and in most ecosystems that have been examined (Roberts, 2008). The tetracycline resistance genes were chosen to cover the three known mechanisms of resistance including efflux [*tet*(B)], ribosomal protection [*tet*(M)], and enzymatic [*tet*(X)] (Roberts, 2005). Sulfonamide resistance genes (i.e., *sul1*, *sul2*, and *sul3*) are often found on transferable plasmids, with *sul1* and *sul2* being the main determinants of resistance in Gram-negative enteric bacteria of clinical origins (Skold, 2000, Perreten & Boerlin, 2003, Binh *et al.*, 2008). s*ul1* was targeted for investigation since it is typically associated with the class 1 integrons (Antunes *et al.*, 2005, Vinué *et al.*, 2010). Even though *intI1* is not an ARG, it can be used as a proxy for ARG contamination because it is linked to antibiotic, disinfectant, and metal resistance genes, resides in a diverse number of commensal and pathogenic bacteria, is often located on mobile genetic elements, and many common forms are xenogenetic (i.e., assembled relatively recently under selection pressures brought upon by human activities) (Gillings *et al.*, 2015).

Relative gene abundances in the agricultural and non-agricultural soils are presented in a heat map (Fig. 2). The normalization of ARGs to the 16S rRNA gene provides an indicator of the proportion of bacteria carrying ARGs, while also correcting for minor variations in sample processing (McKinney *et al.*, 2010). The heat map and Table 1 both show cropland soils having the highest number of gene occurrences for ARGs and *intI1*, as well as some of the greatest abundances. Interestingly, *erm*(B), *tet*(B), and *tet*(X) were primarily detected in the cropland soils, except for one detection of *erm*(B) in soil from a rangeland (Range7) site and three occurrences of *tet*(B) in soil from recreational (Rec8 and Rec9) and rangeland (Range8) sites. Across all land uses, *sul1*, *tet*(M), and *intI1* were the most frequently detected with 57, 43, and 58 occurrences out of 96 samples, respectively (Table 1). The sites with the highest

relative abundances (log_{10} gene copies/16S rRNA gene copies) were all found in cropland soils: *erm*(B), -3.4 (Crop16); *sul1*, -2.5 (Crop4); *tet*(B), -2.3 (Crop16); *tet*(M), -3.4 (Crop33); *tet*(X), -2.8 (Crop38); and *intI1*, -2.5 (Crop36). There was a tendency for the cropland sites to cluster in the dendrogram (Fig. 3) because 38 of 50 sites with the greatest gene abundances were from croplands. In addition, all gene targets were determined to be in significantly greater abundance in the cropland soils when compared to soils from the other land use categories ($P \leq 0.006$; data not presented). Pruden *et al.* (2006) found that sulfonamide and tetracycline ARG levels were greater in environments directly impacted by urban/agricultural activity than in lesser-impacted natural environments.

The above mentioned data suggests that a particular management practice (e.g., tillage, irrigation, fertilization) in croplands could be enriching ARB and/or adding these genes/ARB to the soil. When the cropland gene data was separated by sites that had received dairy manure, dairy wastewater, and/or biosolids at some point in their recent history, it was revealed that all genes, except *tet*(B), were found at statistically greater abundances (7- to 22-fold higher on average) than in soils that were not treated (Fig. 4). To the best of our knowledge, 27 of 46 cropland sites had received organic fertilizer applications the same year or up to several years before we collected the soil samples (Table S1). Since the common denominator in cropland soils of this region is tillage, irrigation, and use of inorganic fertilizers, manure application appears to be the predominant factor contributing to the increased abundance of ARGs and *intI1*. In contrast, Nõlvak et al. (2016) discovered that inorganic nitrogen fertilizer usage alone enhanced the relative abundance of *tet*(A) in an agricultural grassland soil, most likely by stimulating indigenous bacterial populations. In our studies (Dungan et al., 2017; McKinney et al., 2018), use of inorganic fertilizers did not result in ARG increases above those in no-fertilizer control plots, which is supported by results from Lin et al. (2016) and Udikovic-Kolic et al. (2014).

Many studies to date have demonstrated that the use of animal manures in soil enlarges the reservoir of clinically relevant ARGs (Heuer *et al.*, 2011, Ruuskanen *et al.*, 2016), at least for a transient period after application (Marti *et al.*, 2014, Liu *et al.*, 2017). Specific to the region of the present study (i.e., south-central Idaho), Dungan *et al.* (2018)) found that monthly applications of dairy wastewater (straight or diluted to 50%) to a silt loam soil increased the abundance of *intI1*, *erm*(B), *sul1*, and *tet*(M). Only *sul1* was detected in the soil before treatment, while *intI1*, *erm*(B) and *tet*(M) were below detection

limits. In the same soil type, annual applications of dairy manure for fertilizer purposes significantly increased the abundance of *intI1*, *sul1*, *tet*(W), and *tet*(X) (McKinney *et al.*, 2018). When animal manure and wastewater are land applied, ARB and other constituents (e.g., antibiotics, nutrients, metals, disinfectants) are introduced into the soil (Schauss *et al.*, 2009, Ji *et al.*, 2012, Dungan *et al.*, 2018). Furthermore, the accumulation of ARGs in manured soil depends on the input of resistant bacteria from the manure, their horizontal gene transfer to soil-adapted bacteria, and the selective pressure of antibiotic residues and other chemical stressors (Binh *et al.*, 2008, Marti *et al.*, 2013, Udikovic-Kolic *et al.*, 2014, Xie *et al.*, 2018). While manure treatment does indeed influence the occurrence and abundance of ARGs in soil, it is important to note that ARGs were detected at similar levels in non-agricultural soils of this study (i.e., forest, recreational), which supports findings that they are present in background (native) environments (Durso *et al.*, 2016, Rothrock *et al.*, 2016). This information will be useful to those who are trying to understand the spread of antibiotic resistance in agroecosystems, since it is critical to include background sites that lack anthropogenic disturbance for comparative purposes. Normalization of study data to background data can help more accurately determine the impact of agricultural management practices on antibiotic resistance within an agroecosystem (Rothrock *et al.*, 2016).

The correlation analysis (Table 2) shows that the relative abundance of *sul1* and *tet*(M) were highly correlated with *intI1* (r = 0.76–0.77, $P < 0.0001$). A number of studies have found positive correlations between the abundance of the *intI1* and *sul1* (and other ARGs) in soils, which is expected since they are typical components of class 1 integrons (Gillings et al., 2015). In soil amended with cattle slurry or cattle slurry digestate, relative abundances between *intI1* and *sul1* were highly correlated (r = 0.88–0.98, $P < 0.001$) (Nõlvak *et al.*, 2016). Lin *et al.* (2016)) also found a strong correlation between *intI1* and *sul1* (r = 0.97, $P < 0.01$), as well as with *tet*(A), *tet*(G), tet(W) and *sul2* (r = 0.71–0.97, $P <$ 0.01), in a paddy-upland rotational soil. Peng *et al.* (2017)) found that *intI1* was highly correlated with *sul1* (r = 0.99, $P < 0.01$) and other ARGs [*tet*(G), *tetB*(P), *tet*(O), *tet*(W), *sul2*, *erm*(B), and *erm*(F)] (r = 0.74–0.98, $P < 0.01$) in soils under a wheat-soybean rotation. In these latter two studies, the correlations were largely driven by the application of composted chicken manure and swine manure, respectively. Other highly correlated genes in the present study were *erm*(B) and *tet*(X) [r = 0.78, $P < 0.003$] and *tet*(B) and *tet*(X) [r = 0.74; $P < 0.024$] (Table 2). There were also significant correlations between *sul1* and

tet(M) [$r = 0.58$, $P < 0.0004$) and *tet*(M) and *tet*(X) [$r = 0.49$, $P < 0.033$], but the *r*-values were < 0.70 and not considered to be highly correlated. In addition, correlations between the gene targets and soil properties (i.e., total C and N, organic C, pH, and electrical conductivity) in the present study were tested on log transformed data, which were found to be neither highly correlated and significant (data not shown). In a study of archived soils by Knapp *et al.* (2011), some significant correlations were found between ARG levels and geochemical properties, such as pH and heavy metals.

Conclusions

Regions under intensive agricultural production are suspected of contributing to the growing threat of antibiotic resistance, thus understanding the development and spread of antibiotic resistance determinants in agroecosystems is important in protecting human, animal, and ecological health. In this study, cropland soils in south-central Idaho were found to contain a greater frequency and abundance of ARGs and *intI1*, with manure application being the primary reason. Because ARB and ARGs are entrained in manure-derived organic fertilizers, their addition to soils simply increases the total bacterial and gene load. Animal manures and biosolids also contain antibiotics, metals and disinfectants, which could also be influencing ARG levels in the cropland soils. While the occurrence and abundance of ARGs in cropland soils was statistically greater than that in soils from the other land use categories, this does not necessarily mean that an increased rate of horizontal gene transfer from manure-borne to soil bacteria occurred. However, considering the potential health risks caused by ARB, measures to reduce ARB/ARGs and chemical residues in manures prior to their land application should be investigated. In addition to manure application, efforts should also be devoted to determining how other soil/crop management practices, as well as the role of the rhizosphere, influence the abundance and spread of ARGs. Lastly, the results from the present study will be useful to help guide researchers in their selection of background sites during investigations of antibiotic resistance in agroecosystems, as well as those seeking to conduct antibiotic resistance risk assessments.

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Figure Captions

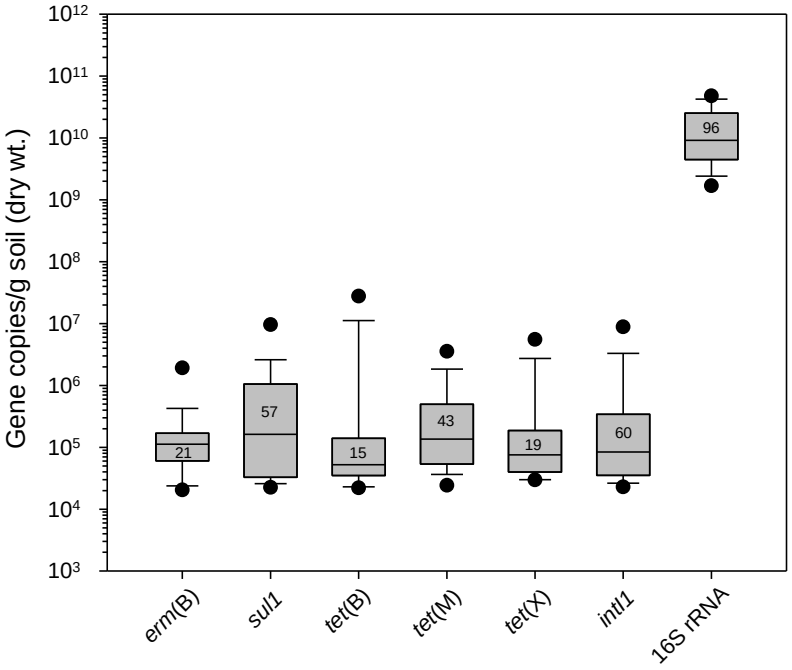

Figure 1. The absolute abundance [gene copies/g soil (dry wt.)] of antibiotic resistance [*erm*(B), *sul1*, *tet*(B), *tet*(M) and *tet*(X)], class 1 integron-integrase (*intI1*), and 16S rRNA genes in agroecosystem soils from south-central Idaho. The horizontal lines in the box plots, from top to bottom including the whisker caps, represent the 10^{th}, 25^{th}, 50^{th}, 75^{th}, and 90^{th} percentiles, while the black circles are the 5^{th} and 95^{th} percentiles. The values inside the box plots are the number of positive gene detections out of a maximum sample size of 96.

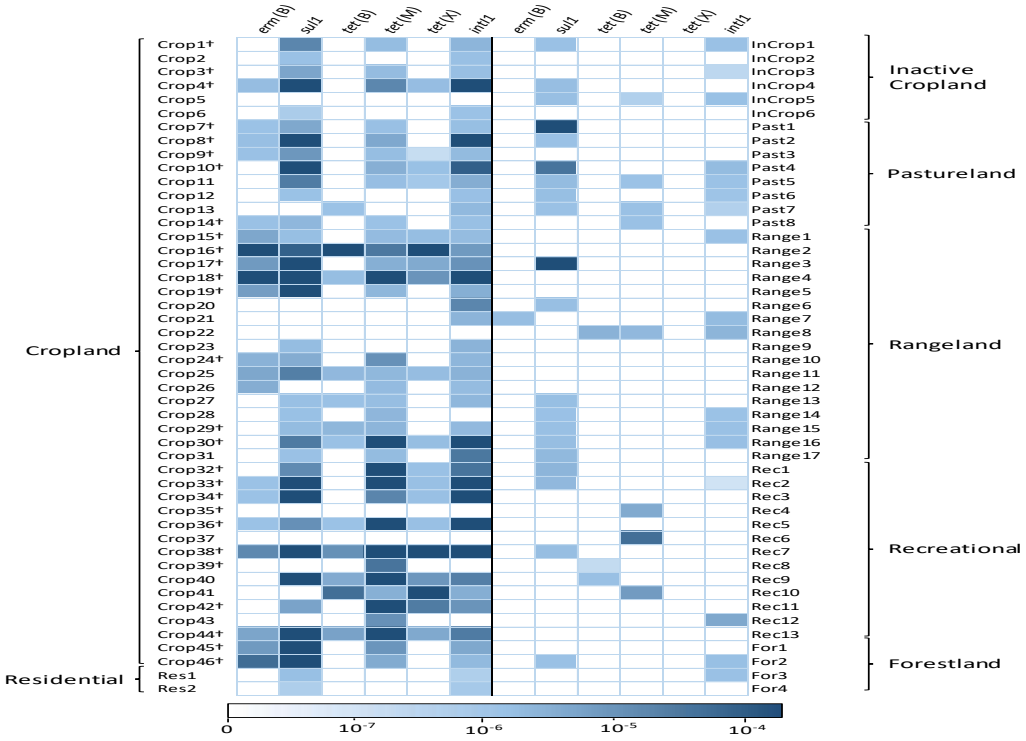

Figure 2. Heat map illustrating the relative abundance of antibiotic resistance [*erm*(B), *sul1*, *tet*(B), *tet*(M) and *tet*(X)] and class 1 integron-integrase (*intI1*) genes in the soils by land use category. The color scale at the bottom indicates the gene abundance from no detection (white) to $\geq 10^{-4}$ gene copies/16S rRNA gene copies (dark blue). †Indicates cropland soils that have a history of receiving dairy manure, dairy wastewater, or biosolids. Detailed information about the sampling sites can be found in the Table S1 (Supporting Information).

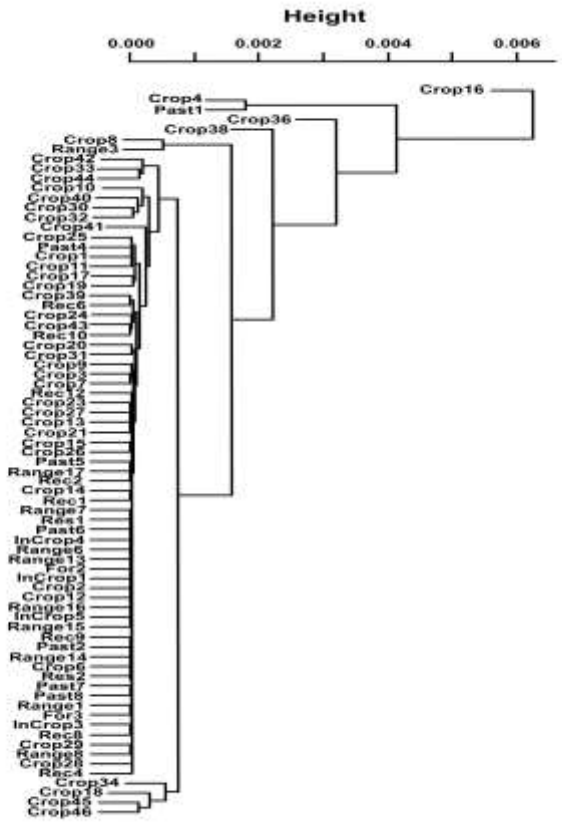

Figure 3. Dendrogram showing clustering for 77 land use sampling sites compared based on the relative abundance (gene copies/16S rRNA genes) of antibiotic resistance [*erm*(B), *sul1*, *tet*(B), *tet*(M) and *tet*(X)] and class 1 integron-integrase (*intI1*) genes. The hierarchical cluster analysis was performed using the "complete" agglomeration method and the square Euclidean distance for distance measures. The descriptor for each line is a code for each land use sampling site: Crop = cropland; For = forest; InCrop = inactive cropland; Past = pastureland; Range = rangeland; Rec = recreational; and Res = residential. Nineteen of the original 96 sampling sites were not included in the analysis because genes were not detected at these sites.

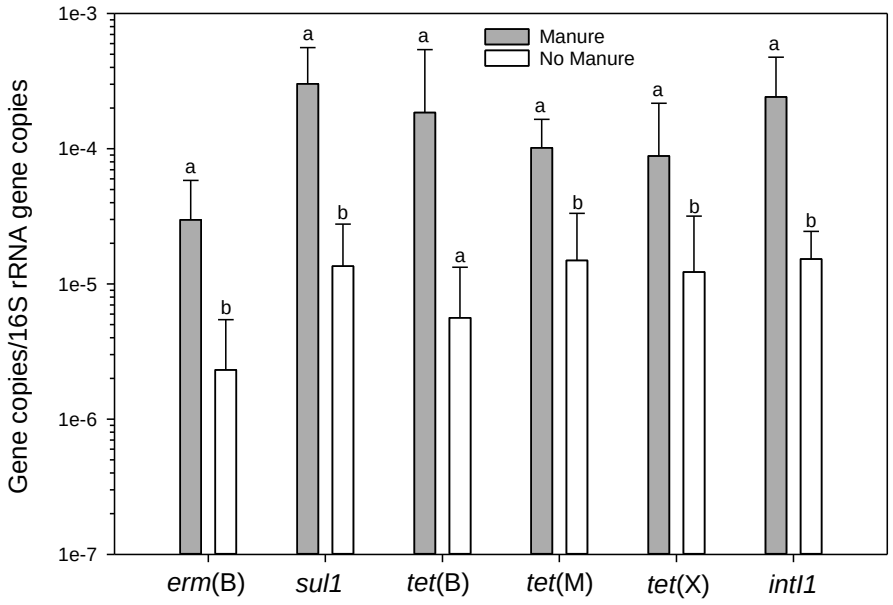

Figure 4. The average relative abundance (gene copies/16S rRNA genes) of antibiotic resistance and *intI1* genes in cropland soils known to be treated with manure, wastewater, and/or biosolids (Manure) and cropland soils that have not been treated (No Manure). Error bars indicate 95% confidence intervals. Columns with different letters (a or b) indicate a significant difference at the 0.05 probability level. Detailed information about the sample sites can be found in the Table S1 (Supporting Information).

Table 1. A summary of ARG and *intI1* gene occurrences and relative abundances (log_{10} gene copies/16S rRNA gene copies) in soils from croplands and all other land uses.

	Cropland			All other land uses		
Gene	(n = 46)	Max	Mean	(n = 50)	Max	Mean
erm (B)	20	-3.4	-4.7	1	-5.3	-7.0
sul1	35	-2.5	-3.7	22	-2.5	-4.0
tet (B)	12	-2.3	-4.0	3	-4.8	-6.4
tet (M)	35	-3.1	-4.2	8	-4.1	-5.5
tet (X)	19	-2.8	-4.2	0		
intI1	39	-2.5	-3.8	19	-4.6	-5.8

Table 2. Pearson correlation analysis of relative ARG and *intI1* gene abundances in the agroecosystem soils from all land uses.

	sul1	***tet* (B)**	***tet* (M)**	***tet* (X)**	***intI1***
***erm* (B)**	0.137	0.756	0.187	**0.783**	-0.188
	0.577	0.082	0.431	0.003	0.416
	19	6	20	12	21
sul1		0.300	**0.575**	0.216	**0.761**
		0.400	0.0004	0.390	<0.0001
		10	34	18	48
***tet* (B)**			0.122	**0.735**	-0.079
			0.705	0.024	0.799
			12	9	13
***tet* (M)**				**0.491**	**0.758**
				0.033	<0.0001
				19	36
***tet* (X)**					0.038
					0.877
					19

In each cell, the upper value is the Pearson correlation coefficient (r), the middle value is the P-value, and the lower value is the number of observations (n). Bold values indicate statistical significance ($P < 0.05$).